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Identification of a novel cephalosporinase (DHA-3) in *Klebsiella pneumoniae* isolated in Taiwan

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ABSTRACT

A strain of *Klebsiella pneumoniae* resistant to cefoxitin and oxyimino-cephalosporins, but susceptible to cefepime, was isolated from an adult patient hospitalised in Taichung, Taiwan. Isoelectric focusing revealed three β-lactamases with isoelectric points of 5.4, 8.2 and 7.9, respectively. Following PCR with plasmid DNA templates and gene sequencing, these enzymes were shown to correspond to TEM-1, SHV-5 and a novel DHA-1-like enzyme (designated DHA-3). The *bla* genes for TEM-1 and SHV-5 were transferable, but the bla_{DHA-3} gene was non-self-transferable in conjugation experiments. All three *bla* genes were successfully introduced by electrotransformation into an *Escherichia coli* recipient (DH5α), resulting in a similar resistance profile to that observed in the original donor strain. Other *K. pneumoniae* strains producing DHA-1-like enzymes have been identified previously in Taiwan, and this report suggests that DHA-type β-lactamases are continuing to emerge in this country.

Keywords Antibiotic resistance, β-lactamases, DHA-type β-lactamases, *Klebsiella pneumoniae*, resistance, Taiwan

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INTRODUCTION

Plasmid-mediated AmpC β-lactamases (21 types) have been detected in clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella* spp. worldwide [1], including Taiwan. These enzymes include CMY-2 in *E. coli* [2] and *Salmonella* Hadar [3], and DHA-1 [4] and CMY-8 [5] in *K. pneumoniae*. Isolates of *K. pneumoniae* producing DHA-1 have disseminated within a university hospital in southern Taiwan [4] and have also been identified at a medical centre in northern Taiwan [6]. DHA-1 was the first inducible, plasmid-encoded AmpC β-lactamase, and was created by mobilisation of the bla_{DHA-1} gene with an upstream regulator *ampR* gene from the *Morganella morganii* chromosome [7,8]. Similarly, DHA-2, a point-mutant derivative of DHA-1, is an inducible, plasmid-located cephalosporinase detected in an isolate of *K. pneumoniae* from France [9]. The present study describes the characterisation of a further novel AmpC β-lactamase in a clinical isolate of *K. pneumoniae* exhibiting resistance to cefoxitin, cefotaxime and ceftazidime.

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MATERIALS AND METHODS

Bacterial strains

K. pneumoniae strain KP3 was isolated in 2000 from an aspirated, purulent specimen from an adult patient with a liver abscess who was hospitalised at Veterans General Hospital, Taichung, Taiwan. *E. coli* J53 (Azi^r) (sodium azide-resistant) and *E. coli* DH5α were used as recipient strains in conjugation and electroporation experiments, respectively.

Antimicrobial susceptibility testing

MICs were determined by the NCCLS macrodilution tube method with Mueller–Hinton broth [10]. Quality control was assured by testing *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The antimicrobial agents investigated included ampicillin, cephalothin, cefoxitin, cefotaxime (Sigma, St Louis, MO, USA), ceftazidime (Glaxo, Greenford, UK),

cefepime (Bristol-Myers Squibb, Princeton, NJ, USA), ciprofloxacin (Bayer, Leverkusen, Germany) and imipenem (Merck Sharp & Dohme, West Point, PA, USA). MICs of cefotaxime and ceftazidime, with or without clavulanic acid (United States Pharmacopoeia Convention Inc., Rockville, MD, USA) 4 mg/L, were compared as a test for the presence of extended-spectrum β-lactamases (ESBLs) [10]. The ESBL phenotype was confirmed by a reduction of ≥ 3 $\log_2$ dilutions in the MICs of either cefotaxime or ceftazidime in the presence of clavulanic acid [10].

Antagonism testing

The modified disk antagonism method [4] was used to detect induction of chromosomal β-lactamases. Disks of inducing agents and disks of cephalosporins were placed on the surface of Mueller–Hinton agar plates, 25 mm apart. The cephalosporin disks (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg) and cefepime (30 μg). Clavulanic acid (10 μg) and cefoxitin (30 μg) disks were used as inducing agents.

Analytical isoelectric focusing (IEF)

Crude protein extracts containing β-lactamases were prepared by ultrasonication, followed by determination of isoelectric points (pIs) by IEF on an ampholin polyacrylamide gel (pH 3.5–9.5) [11]. Gels were developed with 0.5 mM nitrocephin (Oxoid, Basingstoke, UK). Standards of known pI included TEM-1 (pI 5.4), TEM-4 (pI 5.9), SHV-3 (pI 7.0), SHV-1 (pI 7.6), SHV-4 (pI 7.8) and SHV-5 (pI 8.2); pI values for unknown β-lactamases were calculated by regression analysis.

Conjugation and electrotransformation experiments

Plasmid conjugation experiments were performed using *E. coli* J53 (Azi^r) as the recipient [12]. Transconjugants were selected on Luria–Bertani agar plates supplemented with sodium azide (100 mg/L) and cefotaxime (2 mg/L). In addition, cefoxitin (8 mg/L) was added to prevent selection of ESBL-producing transconjugants, and thus to separate an AmpC-encoding plasmid if both ESBL- and AmpC-encoding plasmids were present in the same donor strain. Electrotransformation of a plasmid DNA suspension from *K. pneumoniae* KP3 into *E. coli* DH5α was performed at a pulsed-field strength of 2.5 kV/cm with a 15-μF capacitor and a 335-Ω resistor using an EasyjecT Optima electroporator (Equibio, Nottingham, UK) as described previously [13]. Electrotransformants were selected on agar containing cefotaxime (2 mg/L) or cefoxitin (8 mg/L). Plasmid DNA was extracted from *K. pneumoniae* KP3, *E. coli* J53 transconjugants and *E. coli* DH5α electroporants with a Plasmid Miniprep Purification Kit (Amersham Biosciences, Uppsala, Sweden).

PCR-based amplification of β-lactamase genes and DNA sequence analysis

TEM-, SHV- and AmpC-related genes (including those for DHA-1, FOX-1 and CMY-2) were amplified using specific primers as described previously [2,14–16]. PCR conditions comprised 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension period of 7 min at 72°C. Amplicons were separated by electrophoresis on agarose 1.5% w/v gels and were visualised by exposure to UV light in the presence of ethidium bromide. DNA sequencing was performed on an automated DNA sequencer (ABI PRISM 373; Applied Biosystems, Foster City, CA, USA). Since the set of primers corresponding to the DHA-1 gene gave a positive result, internal primers for putative *hybF*, *ampR* and *orf-1* sequences from the *M. morganii* chromosome were also used in an attempt to determine the genetic context of the cephalosporinase gene [14]. To identify DHA-related integrons, primers P1 (forward, 5′-CGGATGAAGGCAACCCA-3′) and P2 (reverse, 5′-AAGCAGACTTGACCTGATAGT-3′), specific for class 1 integrons, were used in PCRs [17]. Furthermore, a specific pair of primers, 5′-CTTTTGCCCTAGCTGCGGT-3′ (forward) and 5′-CTCACGCCCTGGCAAGGTTT-3′ (reverse), was used to amplify the common region of In6–In7 at bp 3081–3675 [8].

Southern blot hybridisation

Plasmid DNA samples were analysed by electrophoresis at 30 V for 2 h on agarose 1% w/v gels, denatured and then neutralised on Whatman (Maidstone, UK) filter paper saturated, successively, with sodium dodecyl sulphate 10% w/v, denaturation solution (0.5 M NaOH, 1.5 M NaCl) and neutralisation solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) for 15 min each. Plasmid DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) using an electrophoretic transfer cell (Bio-Rad, Hemel Hempstead, UK). A probe for DHA-1 was prepared by random labelling of the 1140-bp bla_{DHA-1} PCR product with digoxigenin (Digoxigenin Labelling and Detection Kit; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The strain of *K. pneumoniae* (KP3) with resistance to cefoxitin and cefotaxime was isolated from a liver abscess aspirate. It remained susceptible to cefepime (0.5 mg/L) and imipenem (0.25 mg/L). The high MICs of cefotaxime and ceftazidime were not reduced by clavulanate (Table 1), suggesting the presence of an AmpC-type enzyme rather than an ESBL [10]. Induction of the β-lactamases of KP3 was demonstrated by the disk antagonism test, which demonstrated blunting of the zones surrounding the cephalosporin disks adjacent to the inducers, including the clavulanic acid and cefoxitin disks (Fig. 1).

IEF of crude extracts of KP3 and *E. coli* DH5α (pKP3) transformants identified three β-lactamases at pIs of 5.4, 7.9, and 8.2. In contrast, the *E. coli* J53 (pKP3-1) transconjugant contained only two β-lactamases with pIs of 5.4 and 8.2. The enzymes with pIs of 5.4 and 8.2 were shown to correspond to TEM-1 and SHV-5, respectively, by amplification of the genes from the plasmid DNA of the

Table 1. MICs of various β-lactams for *Klebsiella pneumoniae* KP3, the *Escherichia coli* DH5α (pKP3) electroporant, *E. coli* DH5α and the *E. coli* J53 (pKP3-1) transformant, as well as pI values of the β-lactamases produced by each strain

	MIC (mg/L)				
Antimicrobial agent	**KP3 pI 5.4, 7.9, 8.2**	**DH5α**	**DH5α (pKP3) pI 5.4, 7.9, 8.2**	**J53(Azi^r)**	**J53 (pKP3-1) pI 5.4, 8.2**
Ampicillin	> 512	4	64	4	128
Cefoxitin	256	4	32	4	4
Cefotaxime	64	0.12	16	0.12	16
Cefotaxime–clavulanate[a]	64	0.25	8	0.12	4
Ceftazidime	128	0.5	32	0.25	32
Ceftazidime–clavulanate[a]	128	1	16	0.25	4
Cefepime	0.5	0.06	0.12	0.03	0.12
Ciprofloxacin	4	0.06	0.06	0.012	0.012
Imipenem	0.25	0.25	0.25	0.12	0.25

[a]Clavulanate was tested at a fixed concentration of 4 mg/L.

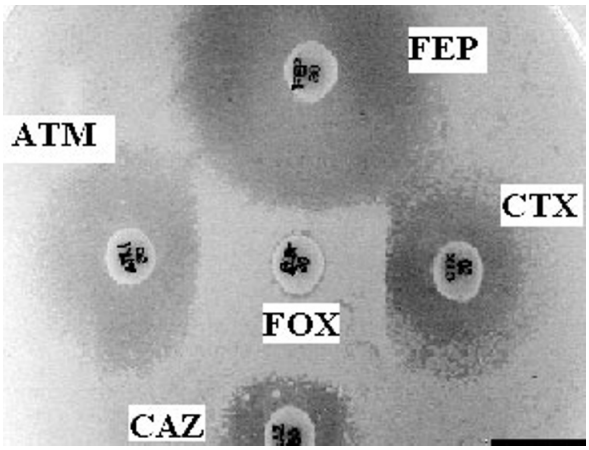

Fig. 1. Example of results obtained with double-disk antagonism tests. ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin.

clinical KP3 strain and the J53 (pKP3-1) transconjugant, followed by DNA sequencing. The naturally occurring SHV-1 enzyme (pI 7.6) of *K. pneumoniae* was not identified on the IEF gel.

The ceftazidime MIC (32 mg/L) for *E. coli* J53 (pKP3-1) was reduced significantly to 4 mg/L in the presence of clavulanic acid (Table 1), confirming the presence of an ESBL (SHV-5). However, clavulanic acid did not reduce the MICs of ceftazidime (128 mg/mL) and cefotaxime (64 mg/L) for the original clinical isolate of *K. pneumoniae* (KP3) (Table 1), suggesting that clavulanic acid itself may act as an inducer of the non-transferred enzyme (pI 7.9), rather than as an inhibitor of SHV-5, thus leading to a false-negative result with the ESBL test for KP3 (i.e., a net effect of no significant reduction in the MICs of ceftazidime and cefotaxime).

Repeated conjugation experiments, regardless of whether cefotaxime or cefoxitin was used as the selecting agent, failed to demonstrate transfer of a plasmid coding for the pI 7.9 β-lactamase. However, this enzyme could be isolated from *E. coli* DH5α (pKP3) electroporants and was subsequently identified as a novel AmpC-like β-lactamase (see below), termed 'DHA-3'. Like the plasmid carrying the $bla_{\text{DHA-1}}$ gene, discovered in strains of *Salmonella enterica* serovar Enteritidis [7,8] and *K. pneumoniae* [4], the $bla_{\text{DHA-3}}$ plasmid was not self-transferable. The *E. coli* DH5α (pKP3) electroporant had a similar resistance profile to KP3 (Table 1).

Using a set of primers specific for $bla_{\text{DHA-1}}$, a 1048-bp PCR fragment was obtained from plasmid DNA preparations of both KP3 and the *E. coli* DH5α (pKP3) transformant. DNA sequencing of both PCR products revealed a deduced protein with only two amino-acid changes (DHA-3) compared with DHA-1 (> 98% similarity; Fig. 2). Repeated PCR and DNA sequencing gave the same sequence for DHA-3, implying that the mutation was not caused by random change. Plasmid analysis by Southern hybridisation with a $bla_{\text{DHA-1}}$-specific probe showed that bla_{DHA} was localised on KP3 and DH5α (pKP3) plasmid bands of >10-kb (data not shown). However, repeated electrotransformation experiments failed to obtain an *E. coli* transformant carrying DHA-3 without SHV-5 and TEM-1. Therefore, precise conclusions regarding the phenotypic effect of the mutations that differentiated DHA-3 from DHA-1 were not possible.

Since DHA-1 and DHA-2 are known to be inducible because of the presence of a reverse transcribed *ampR* gene [7,9], primers targeted to the 3′-ends of this *ampR* gene and the $bla_{\text{DHA-3}}$ genes allowed amplification from the plasmids carried by KP3 and *E. coli* DH5α (pKP3). Comparison of the alignment of the deduced amino-acid sequence of the DHA-3-associated AmpR with that of the AmpR of *M. morganii* revealed 98% identity, with only three amino-acid changes (Fig. 3). The 111-bp inter-cistronic region of *ampC* and *ampR* contained the promoter sequences for *ampC* and *ampR* expression. This region was 100% identical to the corresponding region of the *Salmonella* Enteritidis plasmid that contained $bla_{\text{DHA-1}}$ [7], and was 98% identical to that found in the *M. morganii* chromosome [14]. Based on the inducible β-lactamase activity of KP3 and the *E. coli* DH5α (KP3)

Fig. 2. Alignment of deduced amino-acid sequences of DHA-3 from *Klebsiella pneumoniae* KP3 with those of DHA-1 from *Salmonella* Enteritidis [7] and DHA-2 from *K. pneumoniae* 45956 [9]. Identical amino-acids are marked with dashes. The underlined amino-acids are those that may be involved in the catalytic site of these AmpC enzymes, including the β-lactamase active site S–V–S–K and the conserved triad K–T–G.

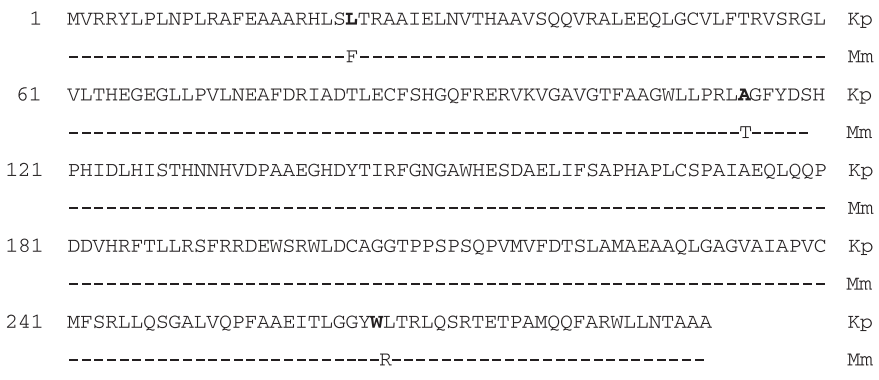

Fig. 3. Comparison of the alignment of the deduced amino-acid sequence of the AmpR protein of *Klebsiella pneumoniae* KP3 (Kp) with that of *Morganella morganii* (Mm) [14].

transformant, as shown by the disk antagonism test, the *ampR* gene should be functional, which may make the DHA-3 enzyme inducible following exposure to agents such as clavulanic acid. This may offset the inhibitory effect of clavulanic acid on the SHV-5 ESBL of KP3, resulting in uninhibited

MICs of ceftazidime and cefotaxime in the presence of clavulanic acid (Table 1).

The sequences surrounding $bla_{\text{DHA-3}}$ were also investigated using PCR to search for *hybF* and *orf-1*. As observed for DHA-2 [9], *hybF* was detected downstream of *ampR*, but *orf-1* was not identified on the plasmid from *E. coli* DH5α (pKP3). These results were also indicative of the *M. morganii* chromosomal origin of $bla_{\text{DHA-3}}$. Although *ampR* and $bla_{\text{DHA-1}}$ were mobilised from the *M. morganii* chromosome into a complex of In6–In7–*sulI*-type class 1 integrons in a strain of *S. enterica* serovar Enteritidis [8], a class 1 integron carrying $bla_{\text{DHA-3}}$ was not identified in KP3, nor was an integron identified in the DHA-2-producing *K. pneumoniae* isolate described previously [9]. Furthermore, PCRs using a pair of primers specific to the common region of In6–In7 [8] did not produce a positive reaction. However, the possibility that point mutations in primer regions have led to these negative results cannot be ruled out.

In conclusion, the present study identified and characterised the third plasmid-mediated cephalosporinase derived from the chromosomally encoded AmpC enzyme of *M. morganii*. Identification of the DHA-1 enzyme in klebsiellae in Taiwan [4,6], together with the discovery of DHA-3 in the present study, highlights the continued emergence of DHA-related enzymes in *K. pneumoniae* in clinical settings in Taiwan, where ESBL-producing strains and other AmpC-producing Enterobacteriaceae have also become prevalent [2–6].

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The nucleotide sequence reported in this paper will appear under GenBank accession number AY494945.

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